## REMARKS

Applicants acknowledge receipt of the Office Action, mailed December 24, 2003. Applicants also acknowledge the withdrawal of the prior Office Action, mailed August 13, 2003. Applicants respectfully request reconsideration of the present application in view of the foregoing amendments and in view of the reasons that follow. This amendment changes and/or deletes claims in this application. A detailed listing is presented, with an appropriate defined status identifier, in relation to all claims that are or were in the application, regardless of whether the claim(s) remain under examination.

Claim 9 is requested to be cancelled. Applicants do not intend to surrender the subject matter contained in the cancelled claim. They retain the right to submit claims directed to this subject matter in the future.

Claims 1-8, 10, and 12-18 are amended. No claims are being added. After the claims are revised as indicated above, therefore, claims 1-8 and 10-25 will be pending. Claims 19-24 are withdrawn. Accordingly, claims 1-8, 10-18, and 25 are presented for consideration.

### Claim Objections

The Examiner raises an objection that pending claim 12 is a substantial duplicate of claim 10 and that pending claim 4 is a substantial duplicate of claim 6. Applicants have amended claims 6 and 12 to return them to their original scope. Applicants respectfully submit that the claims are no longer substantial duplicates.

The Examiner further objected to claim 18 for the misspelling of "least." By amendment, Applicants have corrected the misspelling. Applicants have also corrected the spelling of the term "characterized."

### 35 U.S.C. § 101

On page 3 of the Office Action, the Examiner raises an objection under 35 U.S.C. § 101, contending that claim 1 relates to non-statutory subject matter. Applicants have added the term

"isolated" to claim 1, which now recites "[a]n isolated cell ...." The Examiner has indicated that this addition will obviate the objection.

**35 U.S.C. § 112: Enablement**

With respect to the enablement objection under 35 U.S.C. § 112, first paragraph, the Examiner asserts that Applicants have not shown that the claimed methods produce cells which are pluripotent. Further, the Examiner asserts that "it is known in the art that the expression of Oct4 is not necessarily an indicator of pluripotency."

In response, Applicants submit that it is well-known that *Oct4* expression is indicative of pluripotency. *See, e.g.*, Brehm *et al.*, APMIS. 106(1):114-24 (1998), discussed in the specification at page 28, lines 9-24. In the same vein, Applicants provide a recent article by Flasza *et al.*, *Cloning & Stem Cells* 5: 339-54 (2003), which supports their statement that the disclosed method of reprogramming forms a cell possessing at least one pluripotential characteristic, as evidenced by the expression of *Oct4*. Flasza *et al.* have shown that PEG-mediated fusion of murine embryonal carcinoma ("EC") cell line P19 with a human T-lymphoma cell line (CEM-GFP) resulted in reprogramming of the human somatic cell to exhibit pluripotential characteristics, such as *Oct-4* and *Sox-2* expression.

The field's acceptance of *Oct4* expression as evidence of pluripotency is sufficient to vindicate Applicants' own invocation of *Oct4* expression in the present context. Applicants further submit that, although Monk & Holding (2001) found that *Oct4* is expressed in human tumors, that finding does not detract *Oct4* as a pluripotency marker, contrary to the Examiner's speculation on this subject.

It is widely considered that, during the development of certain malignancies, the malign cells re-establish their pluripotency. Certainly it has long been observed that malignant cells express embryonic markers. Indeed, Monk and Holding themselves state that "cancer cells are also immortal, undifferentiated and invasive. Therefore, it might be expected that cancer cells will express genes in common with these very early embryonic cells, especially genes specifically

associated with deprogramming and return to the undifferentiated and proliferative stem cell state" (page 8085, second column, first full paragraph).

Embryonic stem ("ES"), embryonic germ ("EG"), and EC cells are authentic pluripotent cells ***and*** are capable of tumor formation (see present application, page 10, lines 11 to 13). Therefore, the Examiner is not at liberty to dismiss the significance of genes, such as *Oct4*, that are expressed in a pluripotent cell, merely because those genes also are expressed in a tumor cell. The hallmarks of pluripotency are established anew in a subset of tumor cells. Therefore, the pluripotency of the cells in the specific examples of the present application cannot justifiably be denied by saying that the *Oct4* marker is present in certain tumor cells. Applicants submit that pluripotency has been established in the cells made by the claimed methods using the *Oct4* marker.

The Examiner has noted in the paragraph flanking pages 6 to 7 of the Office Action that the human EC cell line, TERA1, appeared to be unable to reprogram mouse thymocytes, as shown by the lack of expression of mouse *Oct4*. Applicants submit that, as one of ordinary skill in the art would appreciate, the formation of hybrids between two cells of the same species occurs at a frequency as low as $10^{-4}$ to $10^{-5}$, and that formation of hybrids between two cells of different species may occur at an even lower frequency. It is not unexpected that formation of hybrids between a particular pair of cell types may occur at a higher or lower frequency than another pair of cell types. Indeed, cell-cell fusion is a genetically controlled event and may be subject to the same variations as any other Mendelian trait in the genome. Thus, 2102Ep cells may appear to reprogram partner thymocytes more readily than TERA1 cells due to a relative inefficiency of TERA1 cells in forming fusions.

Furthermore, as the majority of cells in fusion experiments remain unfused, one of ordinary skill in the art would appreciate that the assay used to detect reprogramming should be robust in order to compensate for the paucity of fused, and potentially reprogrammed and pluripotent, cells. The amplification of expressed sequences using polymerase chain reaction ("PCR") has a finite capacity to detect rare events in gene expression. Thus, the detection of *Oct4*

expression in fusions between 2102Ep cells and murine thymocytes may have been just above a detectable limit (*i.e.*, extrapolating from a theoretical frequency of fusion, a limit of detecting 1 fused and reprogrammed cell per 10,000 unfused cells). If TERA1 cells yielded fewer fusion events, then the skilled person would appreciate that it is possible that potential reprogramming events might not have been detectable using PCR technology. Other means of measuring reprogramming and pluripotency might show that TERA1 was equally capable of reprogramming thymocytes in fusions.

In the teachings of Flasza *et al.*, *supra*, Applicants also have more recent evidence that the disclosed method of reprogramming to form a cell possessing at least one pluripotential characteristic is not specific for 2102Ep cells. As noted, Flasza and co-workers demonstrated that PEG-mediated fusion of murine EC line P19 to human T-lymphoma line CEM-GFP resulted in reprogramming of the human somatic cell to exhibit pluripotential characteristics. Thus, P19 is a further example of an EC cell, and by extrapolation of ES and EG cells, competent to cause reprogramming of a mammalian somatic cell to exhibit an art-recognized pluripotential characteristic.

For the skilled person, this generalization from EC cells to ES and/or EG cells would seem justified scientifically and, hence, imminently reasonable. Indeed, EC cells are widely thought to represent "caricatures" of ES (and EG) cells. This is evidenced in the mouse and, more recently, with human ES cells, which points to the same conclusion. For example, human EC and ES cells share expression of markers such as SSEA3, SSEA4, TRA-1-60, TRA-1-81, *Oct4* and others. EC cells often appear to lose some of the pluripotential features of ES cells as they adapt to tumour growth. From this point of view, if Applicants had shown directly that ES cells could effect reprogramming, then it might have been reasonable to argue in reverse that EC cells might have lost that capacity. However, given that experiments with oocytes and other cells have indicated that reprogramming capacity is present very early in development, and that we have shown that human EC cells also possess that capacity, it is unwarranted scientifically to argue that ES and EG cells are unlikely to possess such a capacity.

In the paragraph flanking pages 8 and 9 of the action, the Examiner asserts that some of the claims are directed to methods of generating a nuclear transfer (NT) unit, where the nuclear transfer unit is further cultured under conditions to proliferate the NT unit, and that these claims are not enabled as they do not provide steps showing activation of the NT unit which, according to Binnyès *et al.* (2002), must take place. Applicants submit, however, that the present application does not concern an NT unit as referred to by Binnyès *et al.* (2002). Activation is only required where, and inasmuch as, it mimics the act of fertilization brought about by the sperm contacting the egg. In the case of NT, no such sperm exists and thus the activation must be brought about as mentioned by Binnyès *et al.* (2002). Activation to achieve pluripotency in EC, ES, or EG cells has already occurred. For somatic cell reprogramming by an EC, ES, or EG cell, i.e., via the means demonstrated or described in the present application, no activation is required because the new pluripotent cells are not being established through an oocyte. Therefore, the claims of the present application do not need to recite steps of activation as suggested by the Examiner.

In the paragraph bridging pages 9 and 10, the Examiner refers specifically to claim 5 which is directed to a pluripotential cell expressing *Oct4*. The Examiner states that the "specification fails to provide sufficient teachings or guidance to show that the NT [nuclear transfer] unit itself would express Oct-4 as the specification clearly shows that the growth and proliferation of the original unit for 2 days prior to analysis for Oct-4 expression." Applicants submit, first, that the concept of "NT unit" is not directly applicable to the present invention for reasons stated above and, second, that the cell claimed in claim 5 must have at least one pluripotential characteristic by dependency on claim 1. Therefore, a cell that possesses an "NT unit" (in the Examiner's phrase) but that does not have a pluripotential characteristic is <u>not</u> within the scope of the claims, and Applicants should not be required to show support, as suggested by the Examiner, for a cell that is not claimed.

**35 U.S.C. § 112: Indefiniteness**

Claim 1 and its dependents stand rejected in this regard because the Examiner asserts that it is unclear whether claim 1 reads on a cell *in vitro* or *in vivo*. As discussed above, claim 1 is presently amended to recite an isolated cell, so it is now clear that claim 1, and by dependency claims 2 to 18 and 25, refers to a cell *in vitro*.

The Examiner further rejected claims 2 to 8, 10, 12 to 17 and 25 as unclear. These claims have been amended so that they recite "the cell" rather than "a cell." Claim 7 has been further amended to place it in proper Markush form.

The Examiner also rejected claim 3 because it recites a cell which "has the capacity" to proliferate in continuous culture. The Examiner asserts that this phrase is unclear "because it is unclear whether these characteristics actually occur or that the cells could potentially do these described things." Applicants submit that the Examiner's objection is not correct and that one of ordinary skill in the art would clearly understand what is meant by "has the capacity" in the context of ES, EG, or EC cells. Simply put, one skilled in the art would understand, based on the language of the claim and the specification, that the cells must be "immortal" in the sense that they have the ability to proliferate in continuous culture in an undifferentiated state, where such ability would not be present in cells which are not immortal (*see* present application at page 3, line 8, to page 4, line 8, for example). At page 25, line 26, to page 26, line 17, the present application provides conditions under which the skilled person, so informed, could test whether reprogrammed cells are capable of continuous culture.

In this respect, Applicants refer the Examiner to Thomson US Patent No. 5,843,780 (copy enclosed), granted from WO96/22362, which is cited in the present application at page 3, lines 8 to 23. Claim 1 of the '780 patent recites a purified preparation of primate embryonic stem cells which, among other things, "is capable of proliferation in an in vitro culture for over one year." As stated column 12, lines 21 to 26 of US Patent No. 5,843,780: "Immortal cells are capable of continuous indefinite replication in vitro. Continued proliferation for longer than one year of

culture is a sufficient evidence for immortality, as primary cell cultures without this property fail to continuously divide for this length of time (Freshney, Culture of animal cells. New York: Wiley-Liss, 1994)." These references suggest that the phrase "has the capacity," especially when used as it is in the claim, has a well-known meaning in the art. Applicants therefore submit that the claim language is clear.

Applicants have cancelled claim 9, without acquiescing in the rejection to claim 9 and without prejudice to subsequent reinstatement of deleted subject-matter in one or more continuation or divisional applications, for example.

In response to the rejection of claims 13-17, the term "/" where present in "embryonal stem/embryonal germ" has been replaced with the term "or" and where present in "cytoplast/somatic cell fusion" in claim 16 has been replaced with "cytoplast- somatic cell fusion."

**35 U.S.C. § 102**

The Examiner rejects the pending claims under 35 U.S.C. § 102, *i.e.*, that the claims lack novelty over various cited documents. The Examiner notes in the last sentence of page 12 that "the claims recite that the cytoplasm is 'derived from' an embryonal stem cell or embryonal germ cell, and that this language encompasses cytoplasm from any cell, because all cells are derived from embryonal stem cells." The Examiner therefore has taken a broad view on the meaning of "derived from" which is beyond the meaning intended by the Applicant.

In response, Applicants have amended claim 1 by deletion of the term "derived." Claim 1 now recites an isolated cell that comprises at least part of the cytoplasm from a mammalian ES cell or mammalian EG cell combined with a nucleus of a mammalian somatic cell. A basis for this amendment is found, for example, in the application at page 10, lines 19 to 27, page 11, lines 1 to 6, and page 17, line 11, to page 19, line 6. It should now be clear that the claim refers not to cytoplasm from any cell, as held by the Examiner, but to cytoplasm (or at least part of the cytoplasm) specifically from a mammalian ES or EG cell.

The Examiner rejected claims 1-6, 10-12, 18 and 25 as allegedly anticipated by Evans & Kaufman (1981). Evans *et al.* teach the generation of pluripotent cell lines isolated from mouse blastocysts. They do not disclose an isolated cell comprising a cytoplasm from an ES or EG cell combined with the nucleus of a somatic cell as recited in claim 1. In addition, they do not disclose a cell line or cell culture comprising such an isolated cell, methods for preparing such an isolated cell, or a kit comprising such an isolated cell. As such, Evans *et al.* do not disclose each and every claim limitation of claim 1 and therefore do not anticipate claim 1. Since claims 2-6, 10-12, 18 and 25 depend on claim 1, for at least this reason, these claims are patentable over Evans *et al.*

The Examiner rejected claims 1-12, 18 and 25 as allegedly anticipated by Thompson (WO 96/22362). Thompson teaches the isolation and purification of primate ES cells capable of indefinite proliferation *in vitro* in an undifferentiated state. Thompson does not disclose, however, an isolated cell comprising a cytoplasm from an ES or EG cell combined with the nucleus of a somatic cell as recited in claim 1. In addition, it does not disclose a cell line or cell culture comprising such an isolated cell, methods for preparing such an isolated cell, or a kit comprising such an isolated cell. As such, Thompson does not disclose each and every claim limitation of claim 1 and therefore does not anticipate claim 1. Since claims 2-12, 18 and 25 depend on claim 1, for at least this reason, these claims are patentable over Thompson.

The Examiner rejected claims 1, 2, 4-10, 12, 18, and 25 as allegedly anticipate by Campbell (WO97/07668). Campbell is said to teach the reconstruction of mammalian embryos by nuclear transfer, in particular the transfer of a donor nucleus into an enucleated oocyte. Campbell, however, does not disclose an isolated cell comprising a cytoplasm from an ES or EG cell combined with the nucleus of a somatic cell as recited in claim 1. In addition, it does not disclose a cell line or cell culture comprising such an isolated cell, methods for preparing such an isolated cell, or a kit comprising such an isolated cell. As such, Campbell does not disclose each and every claim limitation of claim 1 and therefore does not anticipate claim 1. Since claims 2,

4-10, 12, 18, and 25 depend on claim 1, for at least this reason, these claims are patentable over Campbell. Therefore, none of the cited prior art anticipates the presently claimed invention.

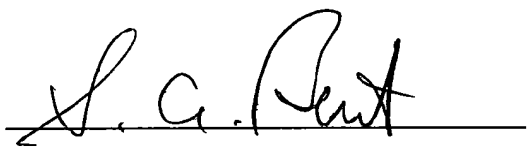
Applicants believe that the present application is now in condition for allowance. Favorable reconsideration of the application as amended is respectfully requested.

The Examiner is invited to contact the undersigned by telephone if it is felt that a telephone interview would advance the prosecution of the present application.

The Commissioner is hereby authorized to charge any additional fees which may be required regarding this application under 37 C.F.R. §§ 1.16-1.17, or credit any overpayment, to Deposit Account No. 19-0741. Should no proper payment be enclosed herewith, as by a check being in the wrong amount, unsigned, post-dated, otherwise improper or informal or even entirely missing, the Commissioner is authorized to charge the unpaid amount to Deposit Account No. 19-0741. If any extensions of time are needed for timely acceptance of papers submitted herewith, Applicants hereby petition for such extension under 37 C.F.R. § 1.136 and authorizes payment of any such extensions fees to Deposit Account No. 19-0741.

Respectfully submitted,

Date 26 April 2004

By 

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# Reprogramming in Inter-Species Embryonal Carcinoma–Somatic Cell Hybrids Induces Expression of Pluripotency and Differentiation Markers

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## ABSTRACT

**Somatic cell reprogramming holds great promise for the development of novel cellular therapeutics. A number of sources of reprogramming potential have been identified, including oocytes, embryonic germ (EG) cells and embryonic stem (ES) cells. However, each of these sources of reprogramming factors is problematic, since they are either not freely available or have special growth requirements. Embryonal carcinoma (EC) cells are another source of pluripotent cells that, unlike ES and EG cells, do not usually require special growth conditions. Since they share many of the key characteristics of ES cells, such as pluripotency, EC cells may provide a readily amenable alternative source of reprogramming factors and could serve as a model for ES cells in this respect. Here we show that mouse EC cells can also function as donors of reprogramming factors. PEG-mediated fusion between murine EC cells (P19) and the cells of a human T-lymphoma cell line (CEM-GFP) resulted in inter-species hybrid colony formation. Colonies of hybrid cells displayed heterogeneity in cellular morphology as well as in their pattern of human gene expression. Expression of two human transcription factors characteristic of undifferentiated pluripotent stem cells, *Oct-4* and *Sox-2*, was detected in the hybrid cells, demonstrating activation of endogenous human markers of pluripotency. Simultaneously, down-regulation of *CD45*, a marker present in lymphocytic cells, was observed in some hybrids. The detection of human specific markers of differentiation, such as *nestin*, *laminin$\beta$1*, and *collagen IV$\alpha$1*, indicates that fusion resulted in reprogramming of the human cells to reflect the differentiation potential of the murine EC partner.**

## INTRODUCTION

THE MOST PROFOUND EVIDENCE of somatic nuclear reprogramming was provided by the live birth of animals cloned by injection of differentiated somatic fetal and adult cell nuclei into eggs (Campbell et al., 1996; Gurdon and Uehlinger, 1966; Wakayama et al., 1998; Wilmut et al., 1997). Pluripotency is also a feature of some somatic cells such as embryonic stem (ES) and germ cells (EG) and, whilst it is not currently feasible to inject somatic cell nuclei into these cells in order to effect reprogramming, it is possible to transfer their pluripotency through the use of cell–cell fusions (Tada et al., 1997, 2001).

Historically, cell–cell fusions have been used to

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demonstrate the phenotypic dominance of one fusion partner (Baron and Maniatis, 1986; Duran et al., 2001; Forejt et al., 1984; McBurney, 1977; McBurney et al., 1978; McBurney and Strutt, 1979; Miller and Ruddle, 1976). Fusions between murine embryonal germ (EG) cells (Tada et al., 1997) or embryonal stem (ES) cells (Tada et al., 2001) and grown under appropriate conditions yielded hybrids with characteristics of the pluripotent partner. Recent work has also demonstrated that spontaneous fusion of murine ES cells with mouse bone marrow (Terada et al., 2002) or neuronal (Ying et al., 2002) derived cells *in vitro* resulted in hybrid cells displaying the characteristics of pluripotent stem cells. In all of these reports, the resulting intra-species hybrid cells expressed markers associated with pluripotency such as the transcriptional regulator, *Oct-4* (Schöler et al., 1989), tissue non-specific alkaline phosphatase, (Berstine et al., 1973) and high levels of telomerase (Bestilny et al., 1996), and were shown to contribute to developing embryonic structures when implanted into blastocysts. Thus, in these intra-specific hybrids, the phenotype of the pluripotent partner appeared to dominate the phenotype of the somatic partner.

Like mouse ES cells, human and murine EC cells are pluripotent (Andrews, 1998; Martin and Evans, 1975; McBurney et al., 1982; McBurney and Rogers, 1982; Miller and Ruddle, 1976). Many EC cells, unlike ES and EG cells, do not require special growth requirements such as feeder layers or addition of survival factors such as LIF, in order to maintain their pluripotency. As such, they would provide a readily amenable and well-characterised source of reprogramming potential and a model for ES cells. In addition to expressing *Oct-4* (Schöler et al., 1989) and other markers of pluripotency, mouse EC cells have been demonstrated to contribute to development upon implantation into murine blastocysts, giving rise to chimaeric animals (Brinster, 1974; Papaioannou et al., 1975, 1978). Similarly, indirect evidence suggests that mouse EC cells also have the ability to reprogram. In early experiments the loss of some thymocyte markers in hybrids with mouse EC cells was observed (Andrews and Goodfellow, 1980; Forejt et al., 1984). Later experiments (Takagi et al., 1983) demonstrated that the normally silent thymocyte × chromosome in EC-thymocyte hybrids was "reactivated," suggesting it had been remodelled to reflect a more developmentally primitive cell. However, while these findings strongly imply that reprogramming by EC cells occurs in somatic cell hybrids, it has not been directly demonstrated that reprogramming of the somatic nucleus genome had occurred in these hybrid cells; it is possible that the somatic genome might remain silent in these hybrids, at least with respect to the expression of genes responsible for pluripotency.

Since ES, EG, and EC cells express endogenous *Oct-4*, *Sox-2*, and other markers of pluripotency, one difficulty in establishing that the somatic partner has been reprogrammed in intra-species hybrids is the availability of appropriate markers and assays. To overcome this problem, other investigators (Tada et al., 1997, 2001; Ying et al., 2002; Terada et al., 2002) made use of transgenic mice carrying GFP, the expression of which was controlled by *Oct-4* transcriptional regulatory elements. Since *Oct-4* is not normally expressed in differentiated somatic cells, expression of GFP indicated that reprogramming had occurred in hybrids formed from thymocytes harvested from these mice and fused to a pluripotent partner. However, although these studies suggested that the thymocyte partner had been reprogrammed, in each case the marker was a transgene randomly integrated into the host genome.

Expression of endogenous markers of pluripotency from the somatic host cells (Pells et al., 2002) would provide more direct evidence of reprogramming and one approach to directly establishing that pluripotency has been reinstated in the somatic cell is to form hybrids from cells of two different species. Cross-species expression of pluripotency markers would indicate that the pluripotent partner had the capacity to reprogram somatic cells in cell–cell fusions. In order to investigate whether EC cells possess reprogramming ability, we fused mouse EC cells to human somatic cells, and looked for evidence of activation of endogenous human specific markers of pluripotency. We here present evidence that EC cells can reprogram somatic cells and that the ability to re-establish markers of pluripotency and differentiation using this technique is conserved across species.

## MATERIALS AND METHODS

### *Cell lines and growth conditions*

Cultures of murine P19; (McBurney et al., 1982) and human NTERA2/D1; (Andrews, 1984; Fogh and Trempe, 1975) embryonal carcinoma cells

were maintained in Dulbecco's Modified Eagles Medium (DMEM, high glucose formulation; BioWhittaker Ltd., Wokingham, UK) supplemented with 10% fetal bovine serum (FBS) (Gibco) and 2 mM L-glutamine (InVitrogen) in a humidified atmosphere of 5% $CO_2$ in air. To passage NTERA2/D1 cells, they were harvested by scraping, and reseeded at a density of $>5 \times 10^6$ cells per 75 $cm^2$, as previously described (Andrews, 1984). CEM-GFP is a clonal cell line derived from the human T-lymphocytic cell line, CEM-C7A, by electroporation (EasyjecT Plus electroporator, Biorad; G. Brady, unpublished data) of the pEGFP plasmid (Clontech) that confers resistance to geneticin and expression of Green Fluorescent Protein (GFP). CEM-GFP cells were maintained in suspension cultures in RPMI 1640 (InVitrogen) supplemented with 10% FBS, 2 mM L-glutamine, and 0.4–0.8 mg/mL geneticin (InVitrogen) at a density of $1–2 \times 10^6$ cells/mL.

*Cell-cell fusion using PEG*

PEG-mediated fusion was performed as previously described (Duran et al., 2001). Briefly, $10^7$ embryonal carcinoma cells were mixed with $10^8$ somatic cells in serum-free medium (DMEM, BioWhittaker Ltd., Wokingham, UK). Mixed cell cultures were collected by centrifugation, the medium was removed from the combined cell pellet and replaced with 0.4 mL of 50% PEG 1500 (Roche Molecular Biochemicals, Mannheim, Germany) or, as a fusion control, 0.4 mL Phosphate Buffered Saline ($Ca^{2+}$, $Mg^{2+}$ free PBS; InVitrogen, Paisley, UK), both pre-warmed to 37°C, and cells were incubated at 37°C for 1.5 min. In order to dilute PEG/PBS and terminate the fusion reaction, 10 mL of serum-free medium pre-warmed to 37°C was gently re-introduced into the mixed cell pellet over five minutes. Cells were gently washed once more in serum-free medium, and finally resuspended in DMEM medium supplemented with 10% FBS and 2 mM glutamine. Cultures of PBS- or PEG-treated cells were plated in four equal aliquots: three aliquots into 75-$cm^2$ flasks and the fourth aliquot into a 150-mm tissue culture Petri dish in order to facilitate cloning of colonies growing in geneticin-supplemented medium.

*Hybrid selection*

To select against parental P19 cells, the medium from the one aliquot of cells plated in 150-mm Petri dish, was removed 24 h after fusion and replaced with fresh medium supplemented with 0.4–0.8 mg/mL geneticin. Prior to replacing the medium the cultures were vigourously washed twice with Dulbecco's PBS to wash away non-fused CEM-GFP cells. Fusion products were incubated in selective medium for 6 days. Colonies of potentially reprogrammed cells were then isolated using cloning cylinders (Sigma Corp., UK), lightly trypsinised and divided into two equal aliquots. Half of each colony was allowed to expand in tissue culture, while the second half was subjected to analysis of gene expression.

*Analysis of gene expression*

Collected colonies were analysed by TaqMan real-time PCR (Applied Biosystems, UK) that detected amplified products through fluorescently tagged gene-specific probes or via SYBR GreenI (Eurogentec Ltd., Belgium). The sequences of primers and probes (where used) are presented in Table 1 and included human (h) and murine (m) *Oct-4*, *Sox-2*, *GAPDH*, and human alkaline phosphatase (*ALPL*), *CD45*, *lamininβ1*, *nestin*, *neuroD1*, *brachyury*, *collagen IVα1*. The primers and probes were designed using Primer Express software (Applied Biosystems) based on 3' untranslated regions of each gene to allow unequivocal discrimination of species homologues.

An aliquot, representing a quarter of the total number of cells in the fusion cultures (approximately $2.5 \times 10^7$ CEM-GFP cells: $2.5 \times 10^6$ P19 cells), was collected at 24, 48, and 72 h or single colonies obtained after 6 or 26 days in selective medium, were harvested as described above and total RNA was isolated using TriReagent (Sigma Corp., UK) according to the manufacturer's instructions. Total RNA samples were resuspended in 10 μL of RNA storage solution (Ambion, UK), and 1 μL of this material was subjected to amplification as detailed in Brady and Iscove (1993). This method results in a collection of amplified polyA cDNAs that accurately represents the abundance of the corresponding mRNA in the starting sample (Al-Taher et al., 2000; Brady and Iscove, 1993; Iscove et al., 2002). An aliquot of this product, undiluted or diluted 100–1,000-fold, was subjected to gene-specific TaqMan real-time PCR (40 cycles), in triplicate, with appropriate specific primers and detection through gene-specific probes (Table 1) or SYBR GreenI, for the desired

TABLE 1. PRIMERS AND PROBES SPECIFIC FOR HUMAN AND MOUSE cDNAs

| *Species/gene* | *Sequence 5'–3'* |
|---|---|
| Human | |
| *Oct-4* | Forward: GGGTTTTTGGATTAAGTTCTTCATTC<br>Reverse: TCACCTTCCCTCCAACCAGTT<br>Probe: CACCCTTTGTGTTCCCAATTCCTTCCTTAG |
| *Sox-2* | Forward: CACACTGCCCCTCTCACACAT<br>Reverse: CATTTCCCTCGTTTTTCTTTGAA<br>Probe: CTCCAGTTCGCTGTCCGGCCCT |
| *GAPDH* | Forward: ACACTCAGACCCCCACCACA<br>Reverse: CATAGGCCCCTCCCCTCTT<br>Probe: TCTCCCCTCCTCACAGTTGCCATGTAGA |
| *Laminin β1* | Forward: CGAAATGCTACAAAATGAAGCAA<br>Reverse: TTGTCTTCATATTTTCTTTCTAAATCTTTGA<br>Probe: AACTCTTTTAGCTCAAGCAAATAGCAAGCTGCAAC |
| *CollagenIVα1* | Forward: TCAGCAGGGCATCGCAT<br>Reverse: AAATGTCATTTCAGGCCTAGTGG<br>Probe: CCGAATCTGCCCTCCTGCGGT |
| *Nestin* | Forward: TGTGGCCCAGAGGCTTCTC<br>Reverse: CAGGGCTGGTGAGCTTGG<br>Probe: CAAAAGCCAGCATGTCACCCTCCCT |
| *NeuroD1* | Forward: GACCTAACTGATATTTCATTATTTGGAATATG<br>Reverse: CCTAAAGGCTAAGCCACTTAAAACA<br>Probe: AGAAAAAAAACACACTGTTTGTACTGCCGTCCAG |
| *Brachyury* | Forward: TTCTGGACCCTGGCAAACAT<br>Reverse: ATGAACCCAACTGTGGAGATGAT<br>Probe: TGGCCCAACCTCACTGACGGTGAA |
| *ALPL* | Forward: CCTGGCAGGGCTCACACT<br>Reverse: AAACAGGAGAGTCGCTTCAGAGA<br>Probe: CTGGGCTCTGAACACACACGCCA |
| *CD45* | Forward: TTGTTGTCAAAAGTATCAAGCAATAAATT<br>Reverse: TGAGCTTATCATGCTGTCTTTACATG<br>Probe: SYBR GreenI (Eurogenetech Ltd.) |
| Murine | |
| *Oct-4* | Forward: GAGGAGGGATTAAAAGCACAACA<br>Reverse: TAAGAACAAAATGATGAGTGACAGACA<br>Probe: CTCCTGATCAACAGCATCACTGAGCTTCTTT |
| *Sox-2* | Forward: TTTTAAAAGATTCGGCTCTGTTATTG<br>Reverse: TTGAAAATGTAGCTGTTATAAGGATGGT<br>Probe: AATCAGGCTCCGAGAATCCATGTATATATTTGAACTAA |
| *GAPDH* | Forward: AACTCGGCCCCCAACACT<br>Reverse: CCTAGGCCCCTCCTGTTATTATG<br>Probe: CATCTCCCTCACAATTTCCATCCCAGAC |

gene targets using an AB7700 "TaqMan" apparatus according to manufacturer's instructions (Applied Biosystems). The human EC cell line NTERA2/D1 served as a control for expression of human markers of pluripotency (h*Oct-4*, h*Sox-2*) or differentiation h*laminin*β*1* (Benham et al., 1983; Wang and Gudas, 1983), h*collagenIVα1* (Benham et al., 1983; Wang and Gudas, 1983), h*nestin* (Przyborski et al., 2000), h*neuroD1* (Andrews et al., 2001), and h*brachyury* (Yamaguchi et al., 1999).

The first cycle of TaqMan at which the instrument can detect release of the fluorescent marker FAM from gene-specific probes or binding of SYBR GreenI to amplified product, is the "threshold" cycle (Ct) for the gene under investigation. In each experiment, the threshold for detection of product was set such that it fell within the linear phase of the amplification reaction. In addition, for each TaqMan reaction a baseline was estimated between cycles 3 and 15. Thus, a Ct value of 15 or less indicated that the reaction was saturated due to over-abundance of the template sequence under investigation. In the experiments described, sample material was subjected to 40 cycles of TaqMan amplification. Control reactions in which no template was added (NTC) resulted in a threshold cycle of 40 (i.e., no detectable output after 40 cycles). Reactions resulting in a Ct of less than 40 indicated the presence of target

cDNA in the sample. In each experiment the data are expressed as the number of cycles that the reaction sample differs from a control in which no template was added (ΔCt; 40-Ct). Each ΔCt increase represents a two-fold increase in the amount of template. Change in the relative expression of the target gene was calculated as $2^{\Delta Ct}$, relative to the no template control, NTC = 1 ($2^{\Delta Ct}$ where ΔCt = 0).

### *Immunostaining*

Cells from hybrid colonies or parental P19 EC cells were plated onto untreated glass coverslips in medium supplemented with 0.8 mg/mL geneticin, where appropriate, and left to attach overnight at 37°C and 5% $CO_2$. Parental CEM-GFP cells were attached to poly-L-lysine–treated coverslips (0.01%; Sigma UK) by brief centrifugation. Cells were fixed in 3% paraformaldehyde in PBS for 20 min at room temperature, washed three times in blocking buffer (5% FBS, 0.1% sodium azide, in PBS), followed by treatment with mouse IgM anti-SSEA1 antibody (Andrews et al., 1982; Solter and Knowles, 1978) diluted 1:5 in blocking buffer, and were incubated for 1 h, 4°C. Cells were rinsed three time with blocking buffer and then incubated in Cy3-conjugated goat-anti-mouse IgM antibody (Jackson Immunoresearch Laboratories, PA), diluted 1:500, for 1 h, 4°C. Finally, cells were washed three times with PBS, mounted in Vectashield (Vector Lab, CA) and viewed by immunofluorescence microscopy (Zeiss). Expression of GFP was examined using a 520-nm wavelength filter, and Cy3 fluorescence was evaluated using a 650-nm wavelength filter.

### *Karyotype analysis*

Karyotype analysis was performed on hybrids approximately 1 month post-fusion essentially as previously described (Duran et al., 2001). To obtain chromosome preparations suitable for G-band analysis, colcemid (InVitrogen) was added to subconfluent, exponential cultures for 2–4 h at a concentration of 0.2 μL/mL. The cells were harvested using trypsin/EDTA (InVitrogen), exposed to hypotonic solution (0.0325 M KCl) for 10 min and fixed (methanol/acetic acid, 3:1). Metaphase spreads were prepared, and for each hybrid, 20–50 metaphases were counted. Images were captured digitally using CytoVision System (version 2.81; Applied Imaging, Newcastle, UK).

## RESULTS

We carried out four independent fusion experiments and three expression assays in order to determine whether reprogramming had occurred. Firstly, we assayed short-term expression of *Oct-4* and *Sox-2*, prior to selection of hybrid colonies in geneticin. Secondly, we allowed colonies of fused cells to grow under selective conditions and assayed for expression of markers indicative of fusion of mouse and human cells. And thirdly, we assayed for specific gene expression indicative of pluripotency and of differentiation in the mouse-human hybrids grown under selection.

The detection level and species-specificity of the primers and probes in the target somatic cell population was established for each of the three genes, h*GAPDH*, h*Oct-4*, and h*Sox-2*. We constructed two reciprocal dilution series consisting of a population of mouse P19 cells ($10^5$) mixed with a diminishing number ($10^5$–0) of human CEM-GFP cells and a population of human CEM-GFP ($10^5$) cells mixed with a diminishing number ($10^5$-0) of murine EC P19 cells (Fig. 1A,B). Using human-specific primers/probes we did not detect the presence of h*Oct-4* or h*Sox-2* in $10^5$ CEM-GFP cells (Fig. 1A), whereas in the reciprocal dilution series, m*Oct-4* was readily detected in as few as 10 P19 cells in a mixed population with $10^5$ CEM-GFP cells (Fig. 1B) using mouse-specific primers. This was in contrast to expression of h*GAPDH*, which we detected in as few as 10 human CEM-GFP cells in a mixed population with $10^5$ mouse P19 EC cells. Thus, probes specific for the human genes did not cross-react with their murine counterparts in as many as $10^5$ (Fig. 1A) nor in as many as $10^6$ P19 cells (data not shown) or vice versa. Expression of h*Oct-4* or h*Sox-2* was detected in human NTERA2/D1 EC cells (Fig. 1A) in as few as 10 cells (data not shown).

### *Short-term expression of Oct-4 and Sox-2 in PEG-treated cultures*

It is well known that hybrids formed between human and mouse cells are karyotypically unstable, and rapidly and specifically lose up to 95% of their human complement of chromosomes unless appropriate selection is applied (Matsuya et al., 1968; Weiss and Green, 1967). Blau et al. (1983) and Baron and Maniatis (1986) addressed this

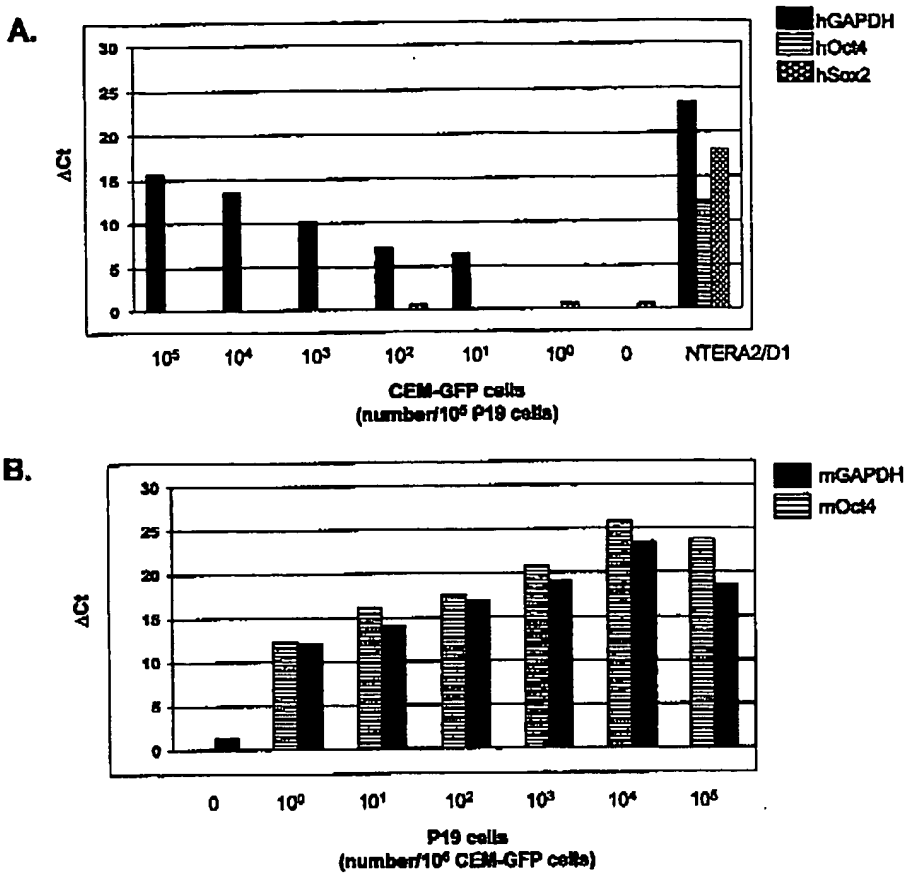


FIG. 1. Expression of *GAPDH*, *Oct-4*, *Sox-2* in CEM-GFP and P19 cells. (A) Mouse EC P19 cells ($10^5$) were mixed with a diminishing number of human CEM-GFP cells ($10^5$–0). The mixed population was assayed for expression of human *GAPDH*, *Oct-4*, and *Sox-2* genes in real-time TaqMan amplification cycles. As a control, expression of the three human genes was also assayed in human EC (NTERA2/D1) cells. (B) Human CEM-GFP cells ($10^5$) were mixed with an increasing number of murine EC P19 cells ($10^5$–0). The mixed population was assayed for expression of murine *GAPDH*, *Oct-4*, and *Sox-2* genes as in A. Note that expression of m*GAPDH* and *mSox-2* is saturated at the equivalent of $10^4$ P19 cells. PolyA-cDNAs were diluted 1,000-fold for each sample.

problem of instability of mouse-human hybrids by assaying for human gene expression 24 h after fusion. We therefore assayed for expression of human *Oct-4* and *Sox-2* during the first 72 h post-fusion. Human *GAPDH*, a ubiquitously expressed housekeeping gene, was used as an indicator of cell number. We noted that expression of h*GAPDH* was relatively high compared to h*Oct-4* and h*Sox-2*; thus, in order to normalize our data, we directly compared expression of the target genes in PEG-treated versus PBS-treated cultures (Fig. 2). In one of three independent experiments assayed in this way, the relative level of h*Oct-4* and h*Sox-2* was seen to increase in the PEG-treated cultures compared to control cultures treated with only PBS at 24 and 48 h (Fig. 2). The relative differences (ΔΔCt) for h*Oct-4* were 2.7 and 10.6 at 24 h (ΔCt 8.4–ΔCt 5.7) and 48 h (ΔCt 10.6–ΔCt 0), respectively, in PEG- versus PBS-treated samples. Similarly, h*Sox-2* expression increased by ΔΔCt 9.5 and ΔΔCt 8.0 over 24 h (ΔCt 9.5–ΔCt 0) and 48 h (ΔCt 8.0–ΔCt 0), respectively (Fig. 2B). This compared with an increase of ΔΔCt of 2.0 and 0.6 over the same time for h*GAPDH* (Fig. 2C). These relative increases in h*Oct-4* and h*Sox-2* had dissipated by 72 h post-fusion (data not shown). The ΔCt, ΔΔCt, $2^{\Delta Ct}$, and fold-change, for each reaction are summarised in Table 2.

No such difference in relative expression of the target genes was observed between PEG- or PBS-treated cultures in the other independent fusion experiments.

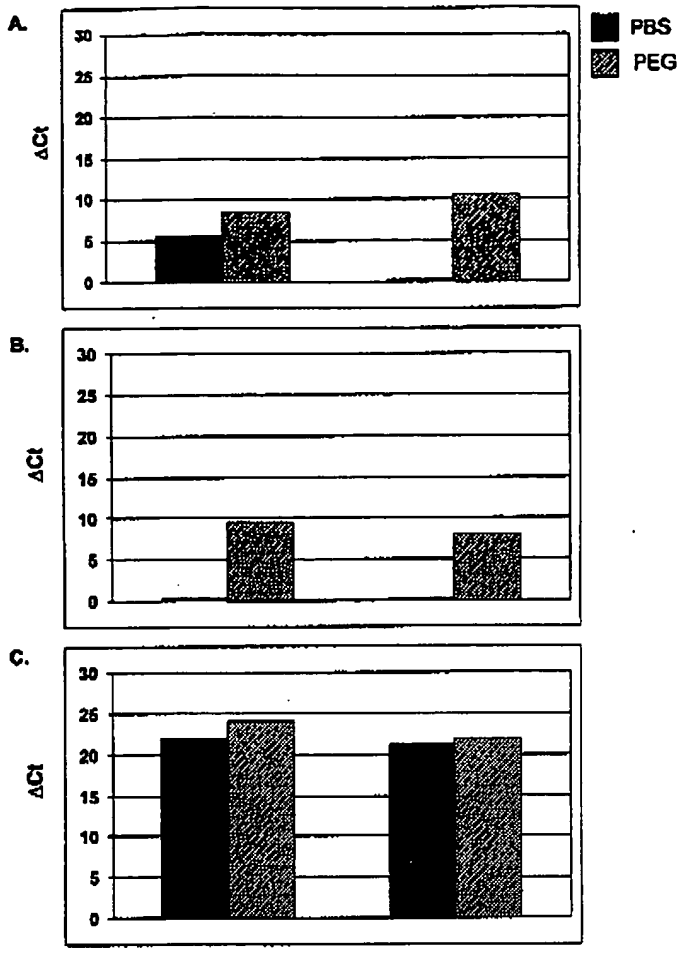


FIG. 2. Short-term expression of human pluripotency markers. A mixed population of P19 and CEM-GFP cells treated with PBS (black bars) or PEG (cross-hatch bars) were assayed for expression of three human genes (*GAPDH*, *Oct-4*, and *Sox-2*), 24 and 48 h post-treatment using TaqMan Real Time amplification. The results are expressed as ΔCt, the number of cycles that expression of the target genes differs in the test samples from a no-template control sample. (A) h*Oct-4*. (B) h*Sox-2*. (C) h*GAPDH*

To test whether PEG treatment per se had an effect on expression of the target genes, we treated CEM-GFP ($10^8$) cells with PEG or PBS. Cells were collected 24 h after treatment and the expression of *GAPDH*, *Oct-4*, and *Sox-2* was determined. There was no detectable difference in expression of the test genes in PEG-treated relative to PBS-treated cells (data not shown).

### *Generation of P19xCEM-GFP colonies*

Detection of short-term inter-species expression of h*Oct-4* or h*Sox-2* was successful in only one of the fusion experiments described above and was further confounded by detectable expression of both genes in the target human population in PBS-treated cultures when assayed from the large number of cells used in these experiments (CEM-GFP $>10^7$ cells). Therefore, an aliquot of cells from each of the fusion experiments was plated into culture medium supplemented with geneticin to select for those EC cells that had successfully fused to CEM-GFP cells. Previous experiments have demonstrated that when non-adherent lymphoid cells and EC (Okuyama et al., 1986), EG (Tada et al., 1997), or ES (Tada et al., 2001) cells are fused, some resulting hybrids grow as adherent monolayers. Thus, non-adherent parental CEM-GFP cells were removed by aspiration of culture medium along

TABLE 2. EXPRESSION OF *hOct-4*, *hSox-2*, AND *hGAPDH* AT 24 AND 48 H POST-FUSION IN ONE EXPERIMENT

| | | 24 h | | | | 48 h | | | |
|---|---|---|---|---|---|---|---|---|---|
| *Gene* | *Treatment* | *ΔCt* | *ΔΔCt* | $2^{\Delta Ct}$ | *Fold-change* | *ΔCt* | *ΔΔCt* | $2^{\Delta Ct}$ | *Fold-change* |
| *hOct-4* | PBS | 5.7 | 2.7 | 52 | 6.5 | 0 | 10.6 | 1 | 1,552 |
| | PEG | 8.4 | | 337 | | 10.6 | | 1,552 | |
| *hSox-2* | PBS | 0 | 9.5 | 1 | 724 | 0 | 8.0 | 1 | 256 |
| | PEG | 9.5 | | 724 | | 8.0 | | 256 | |
| *GAPDH* | PBS | 21.9 | 2.1 | $4 \times 10^6$ | 4.0 | 21.2 | 0.6 | $2 \times 10^6$ | 2 |
| | PEG | 24.0 | | $16 \times 10^6$ | | 21.8 | | $4 \times 10^6$ | |

ΔCt is the number of cycles that a given sample differs from a NTC; ΔΔCt is the number of cycles that one sample differs from a comparator sample (i.e., change in ΔCt relative to control); $2^{\Delta Ct}$ is the change in the number of templates generated relative to NTC.

with any hybrids that did not conform to the adherent EC morphology.

Small colonies of geneticin-resistant, adherent cells were apparent 6 days after plating in selective medium. Three independent experiments yielded 13, 67, and 72, colonies/$10^6$ EC cells plated, respectively, giving an approximate fusion efficiency of $10^{-4}$–$10^{-5}$. This is comparable to, although slightly less than, the efficiency of colony formation seen in intra-species fusions (our unpublished data; and Tada et al., 1997, 2001). In contrast, PBS-treated cultures yielded no colonies of adherent cells in selective medium in any of our fusions.

In order to confirm that the cells growing under geneticin selection were indeed mouse × human hybrids, we examined by immunofluorescence the expression of two specific markers, each expressed exclusively by the mouse or human parental cells. The human CEM-GFP partner is characterized by expression of GFP (Fig. 3A–C), while murine EC P19 cells are immunopositive for SSEA-1, a cell-surface marker expressed on mouse EC cells but not on human CEM cells (Fig. 3D–F). We observed expression of GFP together with expression of SSEA-1 (Fig. 3G–I) only in colonies of cells growing under geneticin selection, confirming that these were hybrids of mouse and human cells.

Fifteen individual colonies were cloned from one of the fusions. Each colony was split in two, one half of which was subjected to real-time PCR analysis of gene expression while the second half was allowed to grow on in tissue culture in the presence of geneticin. Five individual representative colonies exhibiting typical morphology of hybrids growing under selection and for comparison, a colony of parental P19 cells, are shown (Fig. 4A). All colonies contained cells with typical EC morphology: growth in tight clusters within uniform borders, prominent nucleoli, and low cytoplasmic volume compared to nuclear volume (Martin, 1980; Martin and Evans, 1975). At the margins of some colonies, particularly colony 6, we noted cells displaying morphology unlike pluripotent EC cells. While the centre of such colonies contained tightly packed EC-like cells, at the margins we observed cells with a higher nucleus/cytoplasm ratio than EC cells and with a flattened appearance and processes protruding from the cell body (Fig. 4A, arrow). Cells with similar morphology are often observed in EC cell colonies (Andrews et al., 1982, 2001), and it seems likely that these cells arose by spontaneous differentiation of the EC-like cells.

We next surveyed expression of the two markers of pluripotency and *GAPDH* in individual colonies. Every colony expressed human and mouse *GAPDH* and mouse *Oct-4* (Fig. 4B) and *Sox-2* (data not shown) indicating that each colony contained both mouse and human cells and, since they were growing in geneticin, their presence in culture was consistent with hybrid formation. Interestingly, colony 5 expressed little human or mouse *Oct-4* and this might be an example in which some reprogramming, and consequent loss of pluripotency, of the EC cell by the CEM fusion partner occurred as has been previously noted in certain intra-species hybrids (McBurney, 1977; McBurney and Strutt, 1979). Alternatively, these may represent differentiated derivatives of hybrids that initially expressed an EC phenotype. After cloning, this particular colony failed to proliferate further. All individual colonies expressed human *Sox-2* (Fig. 4C) and in addition colonies 1, 6, 7, 8, 9, 10, 11, 14, and 15

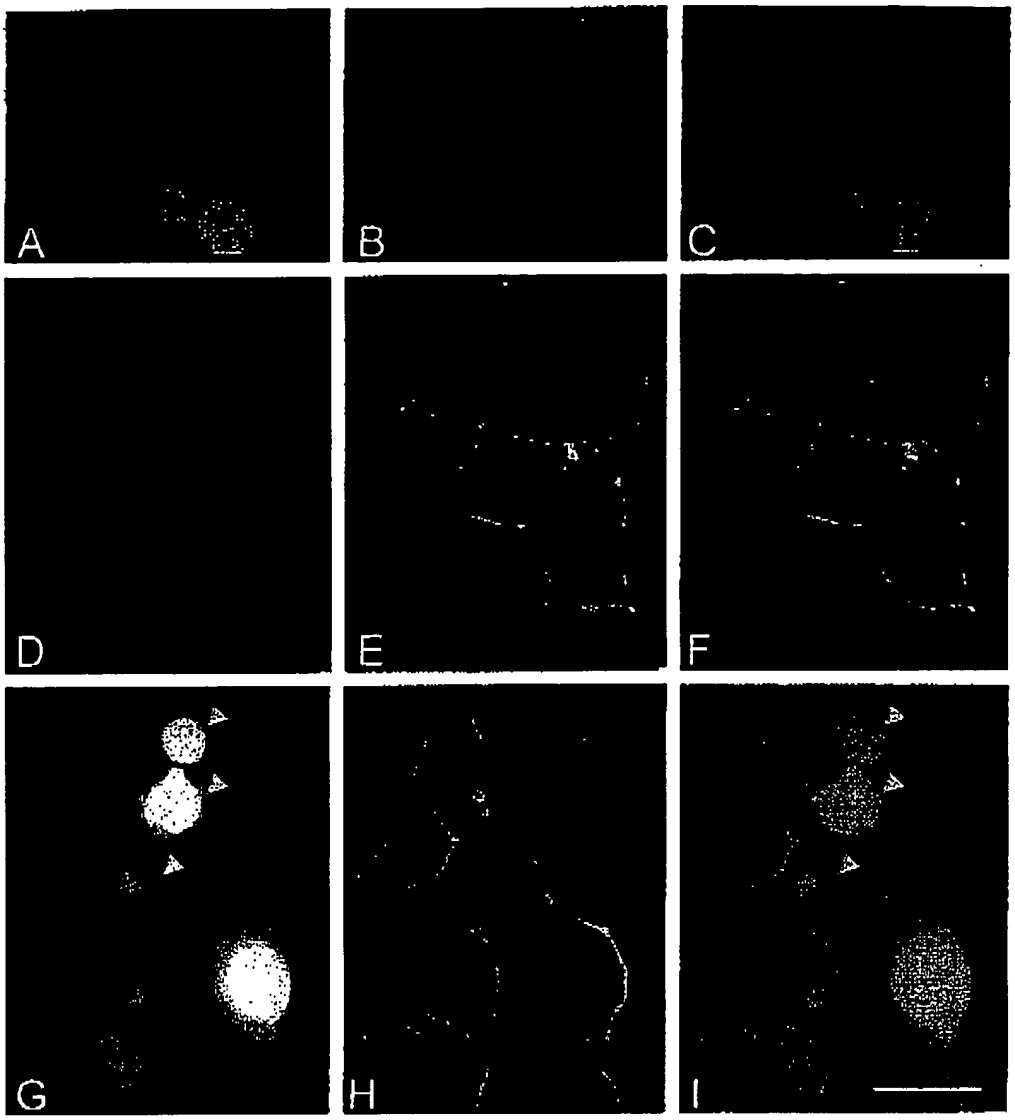


FIG. 3. Expression of GFP and SSEA1 in hybrid cells. Adherent, geneticin-resistant (0.4–0.8 mg/mL) colonies were examined for expression of Green Fluorescent Protein (GFP) and mouse specific EC cell marker SSEA1. (A,B,C) Human CEM-GFP cells. (D,E,F) Murine P19 EC cells. (G,H,I) Adherent hybrid colony. (A,D,G) Expression of GFP. (B,E,H) Expression of SSEA1 detected using mouse anti-SSEA1 antibody. (C,F,I) Merged images (A and B, D and E, G and I, respectively). Arrowheads indicate residual non-fused CEM-GFP cells. Bar = 25 $\mu$m.

also expressed human *Oct-4* (Fig. 4C). We were able to propagate cells from colonies 1, 2, 4, 6, 7, 8, 12, 13, and 14, for at least 26 days when expression of h*Oct-4*, h*Sox-2*, and h*GAPDH*, was assayed again. While expression of h*GAPDH* was detectable in all colonies and h*Sox-2* in all colonies except colony 12, expression of h*Oct-4* was detectable only in colonies 1, 2, 6, and 7. These data are summarized in Table 3. Loss of h*Oct-4* expression and of other human genes at 26 days was probably due to instability of human chromosomes in hybrids. This conclusion was supported by G-band analysis performed on several clones. The tested clones varied in their karyotypes, implying instability. The observed chromosomes in the hybrids were predominantly similar to the mouse parent cells. We observed no identifiable whole or large segments of human chromosomes,

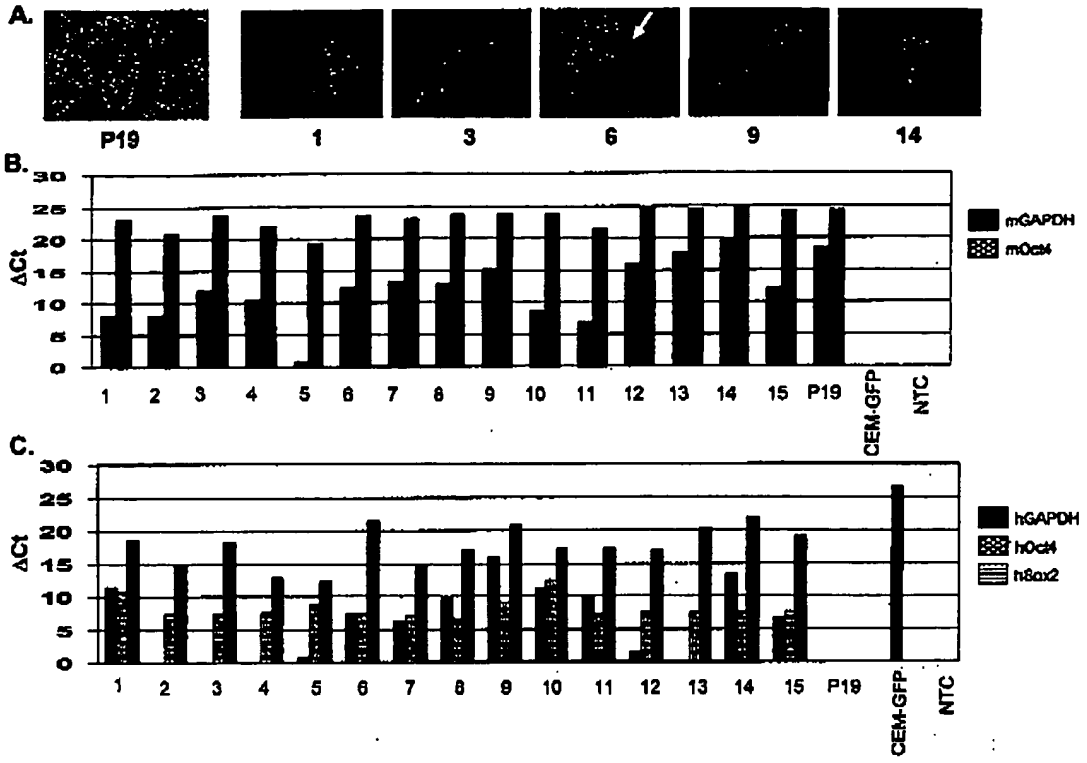


FIG. 4. Morphology and expression of pluripotent genes in hybrid colonies. (A) Examples of five individual hybrid colonies (1, 3, 6, 9, 14) at 6 days post-fusion. The arrow indicates a cell in Colony 6 that appears not to have EC cell morphology. An example of parental murine P19 EC cells is also shown for comparison. Bar = 50 μm. (B) Expression of murine *Oct-4* (cross-hatch bars), and *GAPDH* (black bars) in 15 individual colonies assayed at 6 days post-fusion, and in the parental cell lines, P19 and CEM-GFP. (C) The identical colonies in B and in the parental cell lines were assayed for human *Oct-4* (cross-hatch bars), *Sox-2* (striped bars), and *GAPDH* (black bars) expression. NTC, no template control. PolyA-cDNA templates were diluted 1,000-fold for each sample.

however there were often small unidentifiable chromosome fragments present. We could not exclude the possibility that parts of human chromosomes were present either in the fragments or as small regions inserted into the predominantly mouse chromosomes (data not shown).

Additionally, we investigated *CD45* expression, a gene normally expressed in CEM-GFP cells, in our mouse × human hybrids at 6 days post-fusion. In several colonies (colony 1, 6, 9, 13, and 14), the expression of human *CD45* was down-regulated compared to the level of expression in CEM-GFP cells (Fig. 5). In three colonies (1, 6, 9), the observed down-regulation of *CD45* was not due to complete loss of chromosome 1, where the *CD45* gene resides, since concurrent up-regulation of the human alkaline phosphatase (*ALPL*) gene, also located on chromosome 1, was observed in these colonies. The human-specific primers did not amplify template from greater than $10^4$ P19 EC cells (data not shown).

### *Evidence of differentiation in hybrid cells*

We and others have observed that P19 cultures often contain cells that appear to have a flattened appearance with a higher nucleus/cytoplasm ratio than pluripotent EC cells (Jones-Villeneuve et al., 1982; Martin and Evans, 1975). As noted above some hybrid colonies contained cells that did not wholly resemble an EC morphology, suggesting that some degree of spontaneous differentiation may have occurred in the CEM-GFP × P19 hybrids as it does in a subset of parental P19 cells in tissue culture. Others have described these type of cells as "parietal endoderm" (Roguska and Gudas, 1985; Smith et al., 1987). Parietal endoderm cells have been characterised by their ex-

TABLE 3. EXPRESSION OF HUMAN MARKERS OF PLURIPOTENCY AND DIFFERENTIATION IN CEM-GFP × P19 HYBRIDS

| | *hOct-4* | | *hSox-2* | | *hlamininβ1* | | *hcollagen IVα1* | | *hnestin* | |
|---|---|---|---|---|---|---|---|---|---|---|
| *Days post-fusion* | *6* | *26* | *6* | *26* | *6* | *26* | *6* | *26* | *6* | *26* |
| Colony 1 | ++ | + | ++ | + | − | − | − | − | + | − |
| Colony 2 | − | + | + | + | − | − | + | − | − | − |
| Colony 4 | − | − | + | + | − | − | − | − | − | − |
| Colony 6 | + | ++ | + | ++ | ++ | − | ++ | − | +++ | +++ |
| Colony 7 | + | + | + | + | − | − | +++ | − | − | − |
| Colony 8 | ++ | − | + | + | − | − | − | − | ++ | − |
| Colony 12 | − | − | + | − | − | − | + | − | − | − |
| Colony 13 | − | − | + | + | − | − | − | − | − | − |
| Colony 14 | ++ | − | + | + | − | − | − | − | ++ | − |
| CEM-GFP | − | − | − | − | − | − | − | − | − | − |
| NTERA2/D1 | +++ | | +++ | | +++ | | +++ | | +++ | |

Expression of human pluripotency markers: *Oct-4* and *Sox-2*, and specific differentiation markers: *lamininβ1*, *collagenIVα1* and *nestin* in nine individual colonies at 6 and 26 days post-fusion, and in the parental cell line, CEM-GFP. Human EC cell line NTERA2/D1 was assayed as a control for expression of the markers. (−), ΔCt < 3, (+), ΔCt > 3, (++), ΔCt > 5, (+++), ΔCt > 10, relative to h*GAPDH* expression in the same colony.

pression of *lamininβ1* (Chen and Gudas, 1996) and *collagenIVα1* (Benham et al., 1983; Wang and Gudas, 1983). In addition, under appropriate conditions, P19 cells can be induced to differentiate into skeletal myocytes or neurons (Jones-Villeneuve et al., 1982; McBurney et al., 1982). During the early differentiation of P19 cells induced by retinoic acid (RA), expression of *nestin* mRNA, a marker of primitive neuroblasts, was detected (Gao et al., 2001). Further, differentiation in P19 cells is accompanied by expression of *neuroD1* or *brachyury* when cultures are chemically induced to give rise to neurons or myocytes, respectively. We therefore investigated whether the colonies resulting from our fusion experiments expressed human markers of differentiation. We assayed human gene expression in nine colonies on day 6 and day 26 after fusion (Table 3).

Six days after fusion, expression of human *lamininβ1* was observed in one colony (colony 6), while *collagen IVα1* was detected in two colonies (colonies 6 and 7), and human *nestin* expression was detected in three colonies (colonies 6, 8, and 14). In several of these colonies, for example, colony 6, we detected expression of human *Oct-4* and/or *Sox-2* together with expression of these markers of differentiation suggesting that there existed possible heterogeneity in the fate of individual cells within the colonies. At 26 days post-fusion, expression of *laminin β1* and *collagenIVα1* was no longer detectable in any colonies. However, colony 6 retained expression of *nestin*. We did not detect human *brachyury* or *neuroD1* expression in any colonies at 6 or 26 days post-fusion (data not shown). Furthermore, we did not detect expression of the specific markers of differentiation in parental CEM-GFP cells nor did the primers/probes for the human genes detect

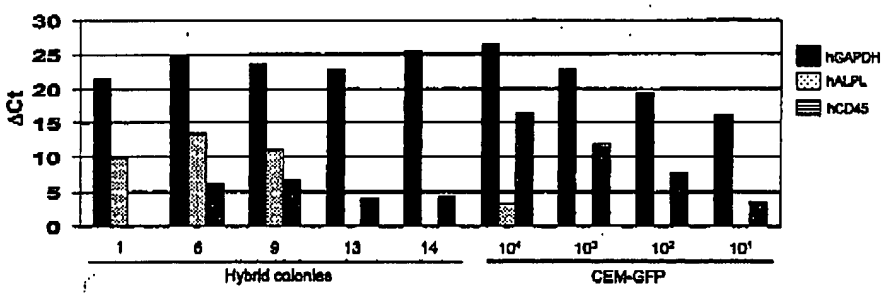


FIG. 5. Down-regulation of human CD45 in hybrid colonies. The expression of human *CD45* (striped bars), human alkaline phosphatase (*ALPL*) (speckled bars), and *hGAPDH* (black bars) in five individual colonies (corresponding to colonies on Fig. 4) assayed at 6 days post-fusion, and in dilution series of parental human CEM-GFP cells ($10^4$, $10^3$, $10^2$, $10^1$). PolyA-cDNA templates were diluted 100-fold for each hybrid sample.

expression of the homologous genes in parental P19 cells. These data are summarised in Table 3. As a further control, we examined expression of these differentiation markers in the human EC cell line, NTERA2/D1 (Andrews, 1984; Fogh and Trempe, 1975), in a culture largely consisting of undifferentiated EC cells. Similar to the P19 × CEM-GFP hybrids, we observed expression of human *laminin$\beta$1*, *collagenIV$\alpha$1* and *nestin*, but no expression of *neuroD1* or *brachyury* in NTERA2/D1 cells, in agreement with previous studies of these cells (Andrews et al., 1983; Gokhale et al., 2000; Przyborski et al., 2000). Since the only source of human gene expression in hybrid cells was the CEM-GFP fusion partner, expression of specific human markers of differentiation strongly suggests that the potential fate of the CEM-GFP partner had been altered to reflect that of its murine EC partner.

## DISCUSSION

Reprogramming of somatic nuclei to reflect a more primitive stage in development has long been established in amphibians (Briggs and King, 1952; Gurdon, 1964) and more recently in mammals (Campbell et al., 1996; Wilmut et al., 1997). Injection of somatic cell nuclei into the permissive environment of the enucleated egg has resulted in live birth of fully developed clones (Campbell et al., 1996; Gurdon and Uehlinger, 1966; Wakayama et al., 1998; Wilmut et al., 1997) suggesting that the egg carries special activity that allows chromatin remodelling to a profound extent. Although it is technically not possible to similarly inject a somatic cell nucleus into a normal cell, it has been possible to demonstrate the reprogramming of somatic cells through fusion with pluripotent ES (Tada et al., 2001) and EG cells (Tada et al., 1997). Although indirect evidence has previously suggested that EC cells have a comparable capacity for reprogramming in cell-cell fusions (Andrews and Goodfellow, 1980; Featherstone and McBurney, 1981; McBurney and Strutt, 1979; Serov et al., 2001; Takagi et al., 1983), we have confirmed using several criteria that indeed this appears to be the case. Firstly, we have observed the expression of human pluripotency markers, *Oct-4*, *Sox-2*, *ALPL*, in hybrids formed between human CEM-GFP and murine P19 embryonal carcinoma cells. Secondly, in some colonies we observed concurrent down-regulation of *CD45*, normally expressed in CEM-GFP cells and other cells of haematopoietic origin. Thirdly, we have detected expression of human markers in some hybrids reflecting the differentiation potential of the murine EC cell that was not available to the human cell prior to fusion. These data strongly suggest that deprogramming (i.e., erasure of the previous developmental fate) and reprogramming occurred in the human cell partner under the influence of the pluripotent partner. Since the reprogramming cells used in these experiments were murine in origin while the reprogrammed cells were human, we conclude that this ability is conserved between species.

Early experiments using fusion of cells to discern dominant and recessive phenotypic characteristics demonstrated that in human–mouse hybrids, the human genome was unstable (Matsuya et al., 1968; Weiss and Green, 1967). Thus, human chromosomes, unless under selection, were frequently and randomly lost in cross-species hybrids. We observed that expression of human genes in our hybrids was unstable. For example, early expression of *Oct-4* was evidently extinguished when assayed some weeks after fusion in some colonies although all colonies assayed retained expression of human *Sox-2*. It seems unlikely that this loss of human *Oct-4* expression was due to complete differentiation of the original colonies since most cells retained an "EC-like" appearance and continued to proliferate and express murine *Oct-4* (data not shown). One explanation is that the human chromosome carrying *Oct-4* was lost during expansion of the colony. Indeed, loss of human chromosomes was observed by karyotype analysis of several of our hybrids. Stable human–mouse hybrids have been described that resulted from fusions between murine muscle and human non-muscle cells (Blau et al., 1983; Blau et al., 1985; Chiu and Blau, 1984). Interestingly, in each instance the resulting hybrids expressed human muscle-specific genes and retained a full complement of human chromosomes however, like the murine muscle parent cells, these hybrids, unlike our hybrids, were non-proliferative and the nuclei did not fuse. The single gene contributed from the CEM-GFP cells that would necessarily be maintained was the gene encoding resistance to geneticin since all cells continued to grow in the presence of selection. One explanation for retention of human *Sox-2* expression in all hybrids is that the plasmid con-

ferring resistance to geneticin may have been integrated on the same chromosome as this gene, but we have not confirmed this. Alternatively, as it has been recently demonstrated that a modest increase in *Oct-4* expression resulted in differentiation of ES cells to primitive endoderm (Niwa et al., 2000), it is feasible that expression from the human homologue resulted in differentiation of some hybrid cells and selection against cells maintaining high levels of human *Oct-4* expression. Pluripotency could still be maintained as long as some cells within colonies retained expression of murine *Oct-4* (Fig. 4B).

Expression of human markers of differentiation were likewise expressed during early outgrowth of colonies but largely lost during subsequent proliferation of the colonies. Random loss of human chromosomes may have accounted for much of the ablation of gene expression. Alternatively, the cells expressing human *nestin*, *collagenIVα1*, or *laminin$\beta$1*, may have differentiated and been diluted in the population by proliferation of those cells retaining EC characteristics. One colony (colony 6) was remarkable in retaining expression of both markers of pluripotency and of human *nestin* over the course of our investigation. The morphology of this colony was similarly notable in its heterogeneity at 6 days after fusion and this heterogeneity appears to have been reflected in its profile of gene expression. Interestingly, none of the colonies expressed *neuroD1* or *brachyury*. Expression of these two genes is observed in P19 cells specifically induced to undergo neuronal or myocyte differentiation, respectively (Farah et al., 2000; Yamaguchi et al., 1999). We postulate that the observed expression of genes associated with differentiation arose through spontaneous differentiation of some cells within the colonies. Spontaneous differentiation of a subset of cells is a common observation in EC cultures in general.

Additionally reprogramming of CEM-GFP cells in three fusion colonies was observed as increased expression of *Oct-4* and *Sox-2* and correlated with reduced expression of *CD45*, a gene normally expressed in lymphocytic and myeloid lineages and in CEM-GFP cells. Simultaneously, expression of human alkaline phosphatase, another marker of pluripotency (Andrews, 1998; Benham et al., 1981) and present on the same chromosome as *CD45*, was observed to increase 6 days post-fusion, suggesting that hybrids were losing T-cell function while gaining features of pluripotency. In the other colonies where no *CD45* or *ALPL* was detected, loss of human chromosome 1 may have accounted for lack of expression of both genes.

We have demonstrated that EC cells have the potential for reprogramming somatic cells to which they are fused. It has been postulated that soluble factors present in enucleated eggs are responsible for reprogramming injected somatic nuclei. Analogous factors are apparently present in ES and EG cells since, as the experiments by Tada et al. (1997, 2001) demonstrated, thymocytes reprogrammed by fusion with these pluripotent cells contributed to development of blastocysts into which they were implanted. Similarly, "transient" heterokaryons of mouse MEL × human erythroid cells analyzed shortly after fusion, were induced to express human adult globin, although the nuclei remained unfused, and the heterokaryons did not undergo replication, strongly suggesting that trans-acting factors present in the murine partner directly "reprogrammed" the human nucleus (Baron and Maniatis, 1986).

Although deprogramming of human somatic cells through fusion with EC cells may yield a viable model of or alternative to ES cells, the resulting cells are karyotypically unstable, and these are unlikely material for use in cell therapies. Furthermore, the use of EC cells per se for the development of cell therapies is precluded by their derivation from teratocarcinomas. Recently, Collas and co-workers have illustrated that various human cell lineages, including fibroblasts, were "reprogrammed" to express activated T-cell and neuronal functions via the transfer of soluble cellular extracts derived from T-cells (Hakelien et al., 2002; Landsverk et al., 2002) or neurons (Hakelien et al., 2002), respectively. We suggest that isolation and transfer of the soluble "reprogramming" factors, which we have here demonstrated are present in EC cells, to somatic target cells could provide a means of developing diploid multipotent stem-like cells for use in future cell therapies.

## NOTE ADDED IN PROOF

During revision of this manuscript, Gurdon and colleagues (Byrne et al., 2003) demonstrated induction of the stem cell marker *oct-4* in murine and human nuclei implanted in *Xenopus* oocytes. Concurrent with expression of this pluripotency marker, the authors observed down-regulation of

the differentiation marker *thy-1* in the murine thymocytes. These observed changes in gene expression occurred in the absence of DNA replication suggesting direct nuclear reprogramming. Their work strongly suggests that somatic nuclear reprogramming is conserved across species.

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